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The cytosolic ribonuclease inhibitor contributes to intracellular redox homeostasis

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Abstract The hypothesis that the cytosolic RNase inhibitor (cRI) has a role in the protection of the cellular redox homeostasis was investigated testing the effects of oxidants and anti-oxidants on normal, primary endothelial HUVE cells, and malignant HeLa cells, before and after their engineering into cRI-deprived cells. We found that cRI plays an important, possibly a key, physiological role in the protection of cells from redox stress, as demonstrated by decreased GSH levels as well as increased oxidant-induced DNA damage in cRI deprived cells.

Keywords: Oxidative stress; Glutathione; RNase inhibitor; Comet assay; RNA silencing

1. Introduction

The cytosolic RNase inhibitor [1,2] is an ubiquitous leucine-repeat protein very rich in Cys residues, which binds with extremely high affinity monomeric RNases of the pancreatic-type, RNase A family, thus fully inhibiting their ribonucleolytic activity.

The hypothesis has been proposed that the physiological role of cRI is that of a "sentry" to protect RNA from RNases which might enter the cytosol [2]. This hypothesis is supported by two lines of evidence: (i) naturally cytotoxic RNases are not inhibited by cRI; (ii) non-cytotoxic RNases strongly inhibited by cRI become cytotoxic when they are engineered into relatively cRI-resistant RNases [3].

Other data however are in contrast with the "sentry" hypothesis, as it has been found that the virtual absence of cRI in HeLa cells, obtained through RNA silencing, does not render these cells sensitive to non-cytotoxic RNases [4]. Moreover, there are RNases which have been shown to be cytotoxic [5], yet they bind cRI with high affinity [6].

Based on the prospect that the biological role of cRI is still unclear, we focused our attention on the possibility that cRI, with its high content of Cys residues, could act also to protect cells against oxidative damage. To verify this hypothesis, based on an early intuition of Hofsteenge and coworkers [7], and in line with recent results [8,9], we propose here a novel experimental approach, based on testing the effects of oxidants and anti-oxidants in cells in which cRI expression has been hindered. The data we obtained indicate that cRI plays the role, possibly physiological, of a defensive system against oxidative stress.

2. Materials and methods

2.1. Materials

RNase A (type XII), diethylmaleate (DEM), *N*-acetyl-cysteine (NAC) and anti-actin antibody were purchased from Sigma. The anti-GAPDH antibody was obtained from Ambion. Complete protease inhibitor was purchased from Roche. Plasmid pU6-cRI-neo^r was prepared as described [4]. RNase A-Sepharose (Amersham Biosciences) was prepared as described by the manufacturer instructions.

2.2. Cell culture and transfection

HeLa cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (HyClone). HeLa sub-clones with undetectable expression of cRI were cultured with the same medium supplemented with 0.6 mg/ml G418 (Invitrogen). HUVE cells (Cambrex) were cultured in EGM-2 Bullet kit (Cambrex). For transfections, cells (1×10^5/well) were grown in 6-well plates and transfected with 1 μg of plasmid DNA in 5 μl of Lipofectine (Invitrogen) as described by the manufacturer instructions. DEM and NAC were added directly to the culture medium as follows: 5 mM NAC (90 min before exposure to DEM), 0.5 mM DEM. After 6 h at 37 °C, cells were collected and lysed by sonication in 0.25 M sucrose, 2 mM DTT in PBS and Complete Inhibitor of proteases.

Several nucleotide sequences from cRI gene (GenBank Accession No. M22414) were tested for constructing the plasmid containing the DNA encoding a siRNA for effective silencing of cRI protein. As detailed in a previous paper [4], and suggested by the plasmid manufacturer (Ambion), the nucleotide sequence 430–449 was identified as the most effective sequence, both for its GC content and its extended distance from the mRNA AUG. The nucleotide sequence for constructing the plasmid encoding a siRNA against the actin gene (GenBank Accession No. NM_001101) included the segment 474–493 from the gene sequence.

2.3. RNase A-Sepharose binding

To evaluate cRI in cell lysates, aliquots of lysates were incubated with RNase A-Sepharose resin for 30 min at room temperature. Unbound cRI was separated by centrifugation at 500*g* for 5 min. The resin was washed twice with 0.5 M NaCl and 2 mM DTT in PBS, eluted by boiling the resin reagent with 1% β-mercaptoethanol, 2% SDS, 0.5 M NaCl and 2 mM DTT in PBS, and run on SDS–

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PAGE. The unbound fractions were precipitated with 50% trichloroacetic acid before loading on SDS–PAGE. Cell lysates, blottings and antisera were prepared as described in [4].

2.4. GSH and thiol assay

Intracellular GSH content was determined as described in [15] with minor modifications: cells were washed twice in PBS, harvested and centrifuged at 1200*g* for 10 min at 4 °C. The pellets were lysed by adding 100 μl of 3% perchloric acid for 15 min and centrifuged at 20000*g* for 10 min at 4 °C. To the supernatant, neutralized with 900 μl of 0.1 M NaH_2PO_4, 5 mM EDTA, glutathione reductase (1 μg/ml) and NADPH (0.21 mM) were immediately added and the mixture was incubated 10 min at 37 °C. GSH content was measured by adding 600 μM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)), and the absorbance of the resulting thionitrobenzoate was read at 412 nm. For measuring protein concentration, the pellets lysed with perchloric acid were resuspended in 1 M NaOH and tested with the Bradford method (BioRad).

Free thiols from small compounds and protein thiols were determined with the colorimetric assay acquired from Molecular Probes following the manufacturer's instructions.

2.5. Comet assay

DNA damage was determined using the Comet assay [16], with modifications. Briefly, cells were washed with PBS, trypsinized, re-suspended in PBS, and combined with LM-agarose at a ratio of 1:8 (cells:agarose). Electrophoretic runs and qualitative and/or quantitative analyses were carried out according to the Trevigen protocol. Quantitative analyses of the results were obtained by using the NIH Image software. Data are reported as the ratio between tail and nucleus areas.

3. Results and discussion

3.1. Silencing of cRI in HUVE and HeLa cells

HUVE cells were transfected with plasmid pU6-cRI-neo^r, containing the DNA template encoding a siRNA which has been shown to effectively and specifically silence human cRI mRNA [4]. The effects of transfection were analyzed at the protein level with an anti-cRI antibody using GAPDH as an internal standard (Fig. 1A). Silencing of the cRI protein was about 45% or 60% 24 or 72 h after transfection (Fig. 1A, lanes 3 and 4).

To verify the specificity of the silencing results, negative and positive control experiments were run on HUVE cells (see Fig. 1, panels B and C). The former were carried out by transfecting cells with an empty vector, deprived of the DNA encoding siRNA. Positive controls were performed by silencing in the same cells actin mRNA. Lysates were tested by Western blotting with anti-cRI antiserum [4] and anti-actin and GAPDH antibodies. In the experiments with the empty vector (see Fig. 1, panel B, lane 1) the levels of cRI, or actin were not affected; in the actin and cRI silencing tests, actin and cRI expressions were specifically silenced by more than 60% (see Fig. 1B, lanes 2 and 3).

It should be noted that stable transfectants of HUVE cells were not obtained, and transient transfections of HUVE cells with pU6-cRI-neo^r were found to be less effective than with HeLa cells. These findings could be related to the much higher level of cRI in HUVE cells, in which 84.2 ± 5 pg of cRI/μg total proteins were found, almost 3.5-fold more cRI than in HeLa cells (see Table 1).

For silencing the expression of cRI in HeLa cells we used stable clones B and O subcloned from the previously investigated clone 11 [4]. About 70% and 95% silencing of the cRI protein was obtained for subclones B and O, respectively (Fig. 1D, lanes 3 and 4). Quantitative data on silencing are tabulated in Table 1.

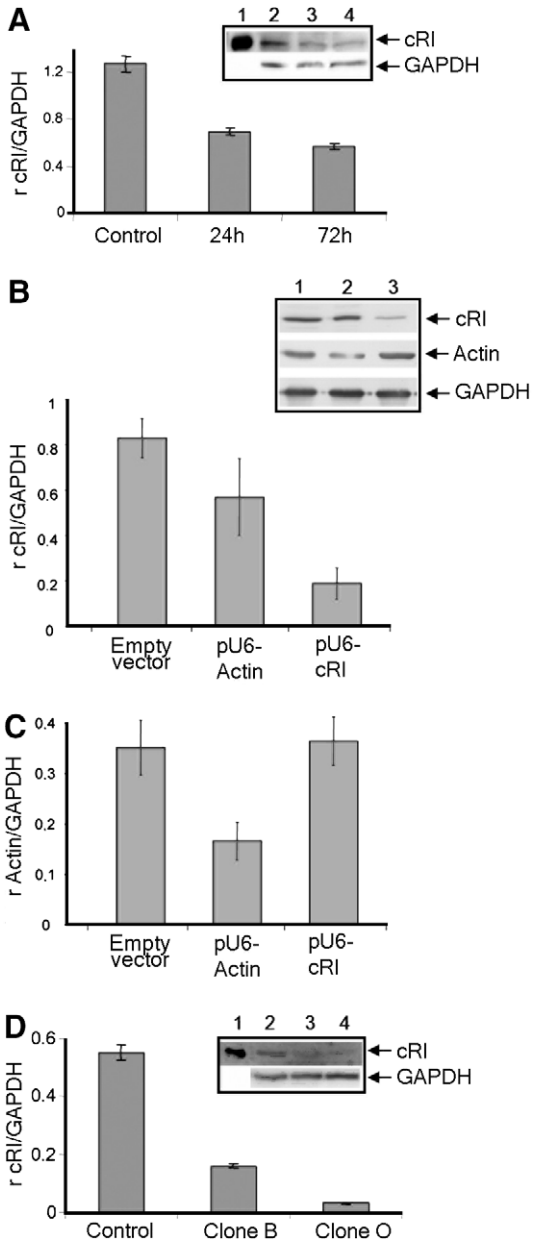

Fig. 1. Silencing of cRI in HUVE and HeLa cells. Western blot analyses of lysates from HUVE (panels A–C) and HeLa (panel D) cells. Histograms were obtained by phosphorimaging analyses using GAPDH as an internal standard. Panel A: lysates from HUVE cells before transfection (lane 2), 24 h (lane 3) and 72 h (lane 4) after transfection with plasmid pU6-cRI-neo^r; lane 1, cRI as a standard. Panel B: lysates from HUVE cells transfected for 72 h with an empty vector (lane 1), the pU6-actin vector encoding the si-RNA against the actin gene (lane 2), the pU6-cRI vector encoding the si-RNA against the cRI gene (lane 3). Western blots were carried out with anti-cRI antiserum (top row), anti-actin antibody (middle row), and anti-GAPDH antibody (bottom row). The histograms reported in Panel B illustrate the cRI levels; those in Panel C illustrate the actin levels. Panel D: lysates from wild-type HeLa cells (lane 2), clone B (lane 3) and clone O (lane 4). In lane 1 cRI as a standard.

3.2. Antioxidant activity of the cytosolic RNase inhibitor

The importance for cRI molecular integrity of the integrity of all its numerous cysteine residues has been ascertained. Either substitution [10] or oxidation [7] of Cys residue(s) can inactivate cRI. To verify the cRI antioxidant role we investi-

Table 1
GSH, thiol and cRI contents in HeLa and HUVE cells

Cell line	pg cRI/μg proteins	nmol GSH/mg proteins	nmol free thiols/mg proteins	nmol protein thiols/mg proteins
HeLa	24.3 ± 1.9	2.1 ± 0.3	25.2 ± 4.5	16.6 ± 5.5
Clone B	7.65 ± 0.5	1.2 ± 0.3		
Clone O	0.07 ± 0.01	0.3 ± 0.1	13.7 ± 0.02	15.3 ± 6.7
HUVEC	84.2 ± 5	0.8 ± 0.3	11.6 ± 6	11.3 ± 6.7
HUVEC cRI$^-$	33.7 ± 1	Not detectable		

gated its redox sensitivity in the living cell upon redox perturbations induced in HeLa cells exposed to 100 or 500 μM diethylmaleate (DEM), a GSH depleting agent. DEM was preferred as an oxidizing agent since it induces an indirect oxidative stress, by forming GSH/DEM complexes in the presence of glutathione transferases [11]. Furthermore, it yields oxidized intracellular conditions much milder than when H_2O_2 is used as an oxidant [12].

The cRI protein was determined in cell lysates by Western blotting with an anti-cRI antiserum, and its activity was tested by analyzing its ability to bind to an RNase A-Sepharose affinity reagent. cRI from untreated cells was found to bind totally to RNase A-Sepharose (Fig. 2, lane 6), whereas lysates from oxidized cells contained: (i) an increasing fraction of cRI which did not bind to the resin, depending on DEM concentration (see Fig. 2, lanes 3 and 4); (ii) a corresponding decreasing fraction of bound protein (see Fig. 2, lanes 7 and 8). Thus, a conclusion could be drawn from these data: that an oxidized intracellular environment, due to DEM-induced GSH depletion, results in cRI inactivation.

These results led us to focus on the hypothesized role of cRI, as part of a thiol pool, in the control of intracellular redox conditions. Therefore, we measured glutathione, the major intracellular scavenging molecule, in HeLa and HUVE cell systems, and compared its levels with the corresponding levels of cRI. In transfected HeLa cells, in which cRI was silenced (clones B and O), a strong decrease in GSH content was found,

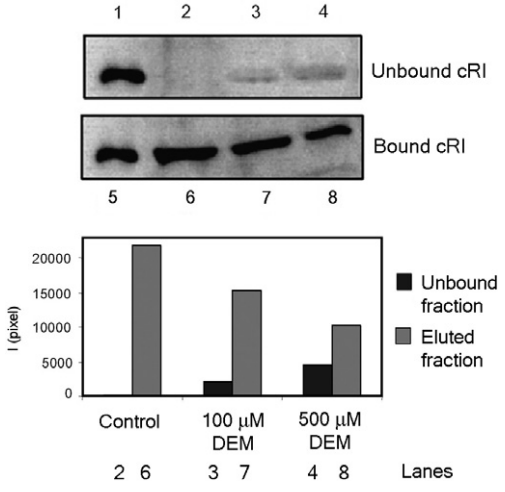

Fig. 2. Effects of DEM treatment on HeLa cells. Western blot analyses with anti-cRI antibody, and corresponding histograms, of cRI levels in lysates of wild type and oxidized HeLa cells analyzed with RNase A-Sepharose as an affinity reagent. In lanes 1 and 5: cRI as a standard. In lanes 2 and 6, cRI protein unbound or eluted, respectively, from the affinity reagent before exposure to DEM; in lanes 3 and 7 and lanes 4 and 8, unbound or eluted cRI, respectively, after exposure to 100 or 500 μM DEM. Intensity of the bands was revealed at the phosphorimager.

with respect to wild type cells (see Table 1). In HUVE cells, with a higher content of endogenous cRI and a low GSH content, upon transfection the GSH level became so low that it could not be detected (see Table 1).

We also determined the levels of free thiols and protein thiols in the cells under investigation. We found (see Table 1) that for HeLa cells, both wild-type and cRI silenced, cRI deprivation engendered a severe drop in the content of small thiol compounds, whereas no significant effects were detected for the level of protein thiols. Given the very low levels of GSH in wild-type HUVE cells, the assay was deemed to be not pertinent in the corresponding cRI-silenced cells.

Hence, in cells deprived of cRI a decrease of the content of GSH and other small thiol compounds occurs, clearly a signal of the altered redox environment resulting from the decrease or absence of an effector, such as cRI, which with its high content of Cys residues contributes to protect the cell from oxidative damage. These results assign to cRI a significant antioxidant role. Furthermore, the finding that wild-type HUVE cells contain high levels of inhibitor, but extremely low levels of GSH, suggests that a balanced pool of thiol-rich molecules, such as cRI and GSH, is present in the cell, whose common role would be an interplay to keep homeostasis through redox "buffering".

3.3. Response of HeLa cells to redox treatments after cRI silencing

It has been established that oxidative stress generates DNA damage [13,14]. Since the redox intracellular environment was found to be altered in cRI deprived cells, we analyzed the effects of cRI silencing on DNA integrity and its interference with GSH homeostasis.

Fig. 3 shows the results of the determination of the GSH content and a Comet analysis of HeLa cells treated, before and after cRI silencing, with DEM as an oxidant, or *N*-acetyl-cysteine (NAC) as a GSH biosynthetic precursor, or a combination of these agents. Even in the absence of any treatment we found that cRI silencing not only led to decreased GSH levels, but also to a marked increase of DNA damage (Fig. 3, panels A and B). A good correlation between DNA damage and GSH levels was observed also when the effects of DEM were analyzed, as DEM induced both a marked GSH depletion (Fig. 3A) and longer comet tails (Fig. 3B). These effects were more marked in cRI-deprived cells. Exposure to NAC, alone or before treatment with DEM, prevented oxidant-induced toxic effects in both wild-type and cRI-deprived cells. These results confirm that cRI-deprived cells are more sensitive to oxidative insults and reinforce the hypothesis of a cellular role of cRI in the prevention of oxidative damages.

3.4. Response of HUVE cells to redox treatments after cRI interference

In HUVE cells cRI deprivation leads to the virtual disappearance of cellular GSH (see Table 1). Thus, the effects of

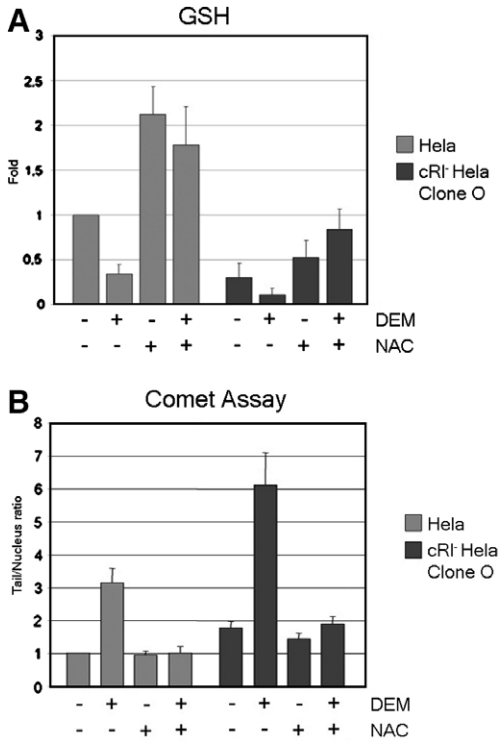

Fig. 3. Effects of redox treatments on GSH content and DNA damage in wild-type (grey bars) and cRI-deprived (black bars) HeLa cells. Cells were treated with either NAC (5 mM), or DEM (0.5 mM) or their combination. Cellular response to these treatments was analyzed measuring GSH content (Panel A) and DNA damage by the comet assay (Panel B).

DEM on the GSH levels of HUVE cells could not be investigated. On the other hand, in HUVE cells cRI silencing was obtained only in a transient mode (see above). Thus, we preliminarily tested the effects of cRI deprivation on cell survival. We observed that 72 h after transfection cell survival was scarcely affected, as more than 70% of cells survived (Fig. 4A). DEM as an oxidant was found to display by itself a cytotoxic effect, but this was significantly higher on cRI deprived cells (Fig. 4A).

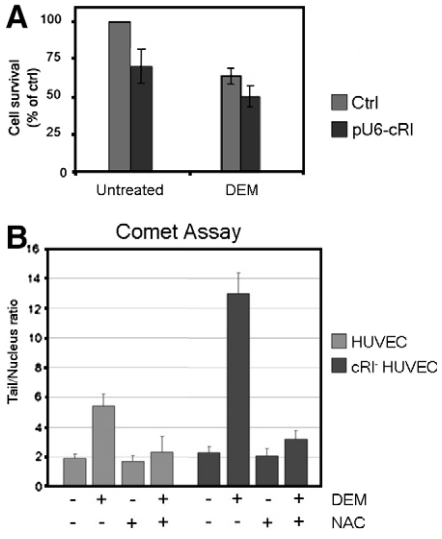

Fig. 4. Effects of redox treatments on wild type and cRI-deprived HUVE cells. Panel A: Cell survival before (black bars) and 72 h after transfection with pU6-cRI-neo^r plasmid (grey bars) in cells untreated or treated with 40 μM DEM. Panel B: analyses of DNA damage by the Comet assay carried out on wild-type (black bars) and cRI-deprived (grey bars) HUVE cells. Cells were treated with DEM or NAC, or their combination, as in Fig. 3.

These findings could be clearly related to the cells redox system [13,14], as DEM was found also to induce a strong increase of DNA damage, especially on cRI-deprived cells, an effect prevented by NAC (see Fig. 4B). These results are in line with the preceding results obtained with the HeLa cells system, and confirm the important role of cRI in the cellular defense against redox stress.

4. Conclusions

As outlined in Section 1, there are doubts that the cellular role of the cytosolic RNase inhibitor is merely that of an RNase inhibitor. It has been suggested instead that this protein, with its high content of cysteine residues (32 over 460 amino acids), has an antioxidant function in the cell, as cells overexpressing cRI become more resistant to H_2O_2 [8,9], and a scavenging activity of cRI on oxygen radicals has been reported [9].

We verified this hypothesis with a radically different strategy, by treating engineered malignant HeLa cells, and primary endothelial HUVE cells, partially or totally deprived of cRI expression, with oxidants or anti-oxidants. Our results demonstrate that GSH levels are decreased in cRI-deprived cells, a signal of an inadequate redox intracellular environment engendered by the lack of cRI as an antioxidant agent. In fact, a general perturbation of the redox balance toward oxidative conditions was confirmed by the decreased thiol levels determined in cRI-deprived cells. As a consequence, cRI-deprived cells were found to become more sensitive to DEM-induced DNA damage.

In summary, the results presented here strongly support the hypothesis that in the cell, besides the tri-peptide GSH, a "giant" GSH is also present, the cytosolic RNase inhibitor, with the role of a "sentry" towards oxidative stresses to maintain a well buffered intracellular redox environment.

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